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## 13. ABSTRACT (Maximum 200 words)

Research to date has resulted in the synthesis of isotope labelled 9-oxononanoate and the development of generic methods for the introduction of a variety of isotopic labels in the 4-hydroxyalkenals. Additional studies demonstrated that 4-hydroxyalkenals could be released from protein sulfhydryl groups. Future studies will investigate whether 4-hydroxynonenal binds to the sulfhydryl groups on human rhodopsin interfering with the visual transduction process.

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# ANNUAL TECHNICAL REPORT

July 1, 1991 until June 30, 1992

## DEVELOPMENT OF METHODS FOR DETECTION OF LIPID PEROXIDATION PRODUCTS IN HUMAN TISSUES GENERATED BY ENVIRONMENTAL TOXINS

- A. The contents of this proposal can be divided into four major parts:
1. Synthesis of isotope labelled 9-oxononanoate, an aldehydic product of lipid peroxidation derived from the  $\alpha$ -end of oxidized fatty acids.
  2. Synthesis of isotope labelled 4-hydroxyalkenals, aldehydic products of lipid peroxidation derived from the  $\omega$ -end of oxidized fatty acids.
  3. Release of 4-hydroxyalkenals from proteins, especially protein sulfhydryl groups.
  4. Use of isotope labelled standards in gas chromatography-mass spectrometry (GC-MS) assays for detection of lipid peroxidation products in human tissues.
  5. New directions.

Progress during the second year of the proposal will be described below for each part.

1. The synthesis of isotope labelled 9-oxononanoate was completed during the first year. Four deuterium labels were put in the molecule, two at the fifth carbon, and two at the sixth carbon. The isotopic purity was 98% when analyzed by GC-MS with negative ion chemical ionization.
2. We have developed generic methods which allow the introduction of a variety of labels in the 4-hydroxyalkenals, including deuterium, carbon-13, tritium and carbon-14. The same method can also be used to synthesize the protonated analog. Furthermore, this synthesis procedure is also generic as far as the size of the molecule is concerned. In other words, the same method can be used to synthesize isotope labelled standards of 4-hydroxyhexenal and 4-hydroxynonenal. This method is different from the synthesis proposed in the original grant, however the generic synthesis has several advantages. The synthesis of 6-D<sub>3</sub>-4-hydroxyhexenal was completed. The synthesis of 6-D<sub>3</sub>-4-hydroxynonenal has been more difficult, since a labelled precursor of this molecule needed to be elongated with three carbons (CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>). This has now been accomplished, and we have just completed this synthesis. The isotope purity of both compounds was greater than 99% when analyzed by negative ion chemical ionization GC-MS.



3. An experienced protein biochemist (Sharon Hapner) has worked on this project since January 1, 1991. Mrs. Hapner has used the conventional assay for titration of protein sulfhydryl groups using Elm's reagent to study the reaction of 4-hydroxyalkenals with proteins. Upon incubation of bovine serum albumin and sodium-potassium-ATP-ase with 4-hydroxynonenal for 1 hour, 33-50% of the sulfhydryl groups disappeared due to reaction with the aldehyde. In addition, we were visited from January until March 1992, by Prof. Werner Siems, from Humboldt University, Berlin, Germany. Prof. Siems is interested in metabolism of 4-hydroxyalkenals. He used a conventional assay to measure the activity of sodium-potassium-ATP-ase, and found that the activity of the enzyme decreases, proportional with an increase of sulfhydryl groups covered with 4-hydroxyalkenals. Subsequently, the aldehydes were released from proteins using reducing agents such as  $\beta$ -mercapto-ethanol. Initial experiments showed that the proteins sulfhydryl groups can be recovered, but in case of sodium-potassium-ATP-ase, the activity of the enzyme is permanently lost. We assume that the disulfide bridges are broken under the reducing conditions used, which may contribute to permanent loss of enzyme activity.

During the third year, we plan to use the technique for release of aldehydes from proteins simultaneous with the extraction and derivatization of aldehydes when tissue samples are analysed by GC-MS. That would make it possible to obtain more accurate values for the content of 4-hydroxyalkenals in tissues.

4. We started some of this work during the first year. We looked for a source of tissue rich in  $\omega$ -3 fatty acids, since their oxidation and breakdown is known to generate 4-hydroxyhexenal, from which the tri-deuterated standard which first was available. The sensory part of the eye, the retina is very rich in  $\omega$ -3 fatty acids, and the PI has much experience working with rat and bovine retina. 3 years ago, when the PI moved to MSU, a collaboration was established with the Montana Eye Bank Foundation (MEBF). The MEBF is able to provide up to 500 human eye samples per year. Initial experiments showed that about 0.5 nanogram of 4-hydroxyhexenal is present in a human retina. This is a very small amount, and we hypothesize that this finding can be explained by the procedures that we used to avoid post-mortem oxidation, and the presence of high levels of anti-oxidants in the human eye. During the second year, we have not worked in this area, since we needed to complete the synthesis of labelled 4-hydroxynonenal to continue to work in this area. We expect to resume these analyses since deuterated 4-hydroxynonenal is now available.
5. New directions were described in detail in the previous annual technical report, including work on human rhodopsin, and measurement of xanthine oxidase and selenium in human eyes. We plan to proceed with experiments incubating human rhodopsin with 4-hydroxynonenal, to investigate whether its binding to the sulfhydryl groups interferes with the visual transduction process.

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Another question we asked was "What is the source of free radicals in the human retina?" Since we were able to detect 4-hydroxyhexenal, it was decided to investigate what causes initiation of lipid peroxidation. A medical student, Stephen J. Mest, from the University of Washington spent two months (summer of 1990) in the lab with support from the University of Washington's Medical Student Research Training Program. We decided to investigate whether the enzyme xanthine oxidase is present in human eyes samples. Xanthine oxidase converts hypoxanthine to xanthine and xanthine to uric acid in the presence of oxygen, thereby producing the superoxide radical. This mechanism has been implied in ischemia reperfusion injury to tissues. We setup the conventional fluorometric assay for xanthine oxidase, and found that a commonly used electron acceptor (2,6-dichlorophenolindophenol) is also a competitive inhibitor for the enzyme. A manuscript describing these results has been published in *Free Radical Biology & Medicine*. Mr. Mest was able to return for two months (summer 1991). We modified the assay to use a different electron acceptor and performed measurements of xanthine oxidase in human eye samples. Unfortunately, it was still not possible to measure the enzyme activity with the improved fluorometric method. A new undergraduate student, Nora Fox has recently joined my group. Mrs. Fox will try antibodies against xanthine oxidase to detect and localize the enzyme in eye tissues. This immunological technique is even more sensitive than the fluorometric method. The antibody will be provided by Dr. G. Bulkley, John Hopkins Univ., Baltimore.

The work on selenium by Mrs. Kosted is under consideration for publication in *Investigative Ophthalmology & Visual Sciences*. The results were described in the previous annual technical report.

## B. Publications.

1. Spectrophotometric quantitation of rhodopsin in the human retina. van Kuijk, Lewis, Buck, Parker and Klinger. *Invest. Ophthalmol. Vis. Sci.* 32: 1962-1967, 1991.
2. Photolysis intermediates of human rhodopsin. Lewis, van kuijk, Thorgeirsson, and Klinger. *Biochemistry*, 30: 11372-11376, 1991.
3. 2,6-Dichlorophenolindophenol is a competitive inhibitor for xanthine oxidase and is therefore not usable as electron acceptor in the fluorometric assay. Mest, Kosted and van Kuijk. *Free Radic. Biol. Med.* 12: 189-192, 1992.
4. Fatty acid composition of the human macula and peripheral retina. van Kuijk and Buck. *Invest. Ophthalmol. Vis. Sci.*, In Press. Vol 33. 1992.
5. Selenium content of the human retina and retinal pigment epithelium. Kosted, Buck, and van Kuijk. *Invest. Ophthalmol. Vis. Sci.*, submitted, 1992.